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Role of the *DRM* and *CMT3* Methyltransferases in RNA-Directed DNA Methylation

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Summary

RNA interference is a conserved process in which double-stranded RNA is processed into 21–25 nucleotide siRNAs that trigger posttranscriptional gene silencing. In addition, plants display a phenomenon termed RNA-directed DNA methylation (RdDM) in which DNA with sequence identity to silenced RNA is de novo methylated at its cytosine residues. This methylation is not only at canonical CpG sites but also at cytosines in CpNpG and asymmetric sequence contexts. In this report, we study the role of the *DRM* and *CMT3* DNA methyltransferase genes in the initiation and maintenance of RdDM. Neither *drm* nor *cmt3* mutants affected the maintenance of preestablished RNA-directed CpG methylation. However, *drm* mutants showed a nearly complete loss of asymmetric methylation and a partial loss of CpNpG methylation. The remaining asymmetric and CpNpG methylation was dependent on the activity of *CMT3*, showing that *DRM* and *CMT3* act redundantly to maintain non-CpG methylation. These DNA methyltransferases appear to act downstream of siRNAs, since *drm1 drm2 cmt3* triple mutants show a lack of non-CpG methylation but elevated levels of siRNAs. Finally, we demonstrate that *DRM* activity is required for the initial establishment of RdDM in all sequence contexts including CpG, CpNpG, and asymmetric sites.

Results and Discussion

The phenomenon of RdDM was first observed when RNA viroids were seen to cause de novo cytosine methylation of homologous genomic DNA sequences [1]. RdDM affects cytosines in all sequence contexts including CpG, CpNpG (N is A, T, C, or G), and asymmetric (CpHpH, where H is A, T, or C) [2]. When RdDM is directed to a promoter sequence it can cause transcriptional gene silencing [3–5].

A two-component transgene system (H/K) has been utilized to study the process of RdDM [3]. A silencer transgene, termed H, consists of the 35S promoter driving the expression of an inverted repeat of the *NOPALINE SYNTHASE* promoter (NOSpro) sequence. Double-stranded RNA from the H transgene is processed into 21–24 nucleotide small interfering RNAs (siRNAs). A second unlinked target transgene, termed K, contains NOSpro driving expression of a kanamycin resistance gene (*NPTII*) and a second cassette with NOSpro driving expression of *NOPALINE SYNTHASE*. The presence of the H transgene causes RdDM and transsilencing of the NOSpro sequences present in the K transgene, causing transcriptional silencing of both the *NPTII* and *NOPALINE SYNTHASE* genes. The expression state of *NPTII* is monitored by testing whether the seedlings are resistant to kanamycin, and the expression state of the *NOPALINE SYNTHASE* gene is assayed by measuring the levels of nopaline present in tissue extracts. The silencing of the NOSpro target sequences is associated with heavy methylation at all CpG, CpNpG, and asymmetric sites [6].

Arabidopsis thaliana has at least three classes of DNA methyltransferase genes that are possible candidates for controlling RdDM: the *MET* class, the *CMT* class, and the *DRM* class [7]. *MET1*, like its mammalian homolog *Dnmt1* [8], encodes the major *Arabidopsis* CpG maintenance methyltransferase [9–12]. In the H/K system, a weak *met1* allele was shown to be defective in the maintenance of transcriptional gene silencing [6]. However, the effects of *met1* were not apparent in the first generation in which *met1* was homozygous and instead showed progressive and stochastic effects as the plants were inbred. Southern analysis of the NOSpro sequence with methylation-sensitive restriction enzymes showed a decrease in CpG methylation, consistent with the role of *MET1* in maintaining CpG methylation. Similarly, in a different RdDM system where a 35S:GFP transgene was methylated and silenced by homologous RNA virus sequences, CpG methylation of the 35S promoter sequence was heritable in the absence of RNA trigger and was dependent on the activity of *MET1* [4]. However, suppression of *MET1* activity did not block the establishment of RNA-directed CpG methylation in this system. These results suggest that *MET1* is important in the maintenance of gene silencing that is caused by RdDM but probably not in the initiation of RdDM. *CMT*-like genes are specific to the plant kingdom and encode methyltransferase proteins containing a chromodomain [13]. *Arabidopsis CMT3* loss-of-function mutants show a large decrease in CpNpG methylation and more subtle and locus-specific effects on asymmetric methylation [12, 14–16]. The *DRM* genes share homology with mammalian *Dnmt3* genes that encode de novo methyltrans-

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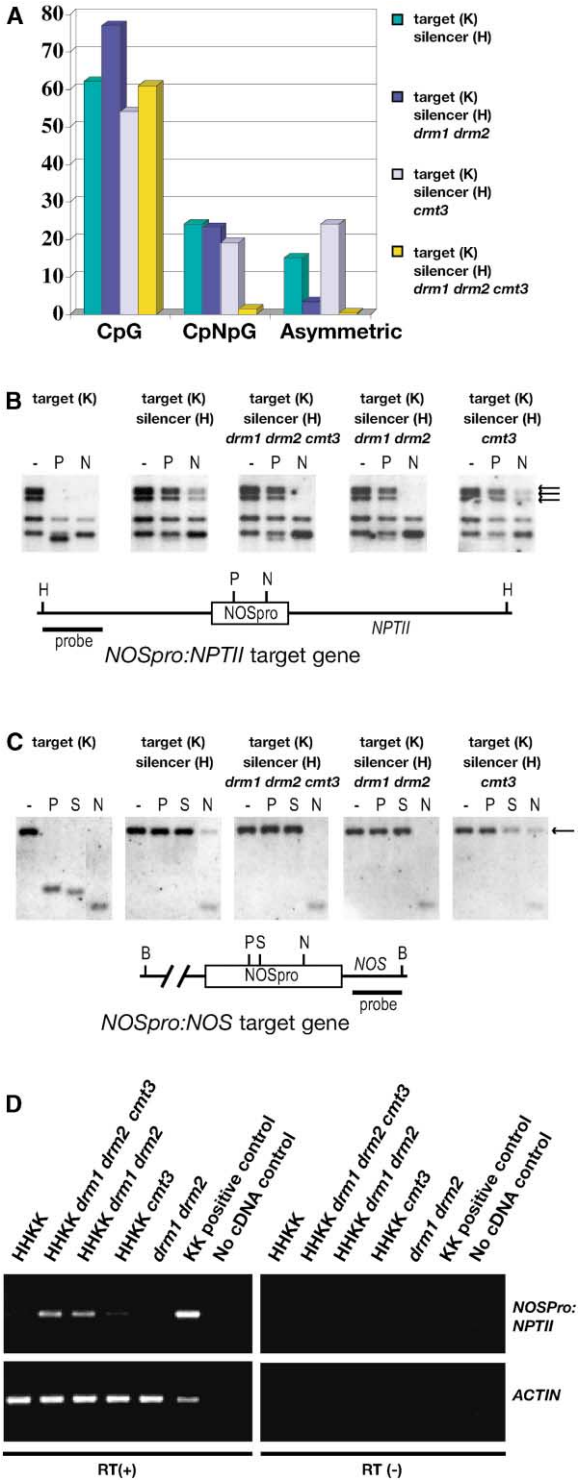

Figure 1. Effect of *cmt3* and *drm* Mutations on Maintenance of Target *NOS* Promoter Methylation and Silencing

(A) Histograms represent percent methylation in different sequence contexts as determined by bisulfite genomic sequencing of *NOSpro:NPTII* (details in Table S1).

(B and C) Target NOSpro methylation was determined by Southern blot analysis for the *NOSpro:NPTII* target gene (B) and the *NOSpro:NOS* target gene (C) with methylation-sensitive restriction enzymes as shown in the maps. In (B), genomic DNA precut with the methylation-insensitive enzyme HindIII (H) was further digested with Psp1406I (P) indicative for CpG-methylation and NheI (N) indicative for CpNpG/CpHpH methylation. Multiple bands in the H cut DNA ("−" lanes) demonstrate that more than one T-DNA copy is present at the target transgene locus. In the unsilenced and unmethylated state (first panel), the upper three bands (marked by arrows) shift to smaller sizes upon digestion with P and N. In the presence of the silencer (H) transgene (second panel), cleavage at these sites is largely blocked due to acquired methylation. The *drm1 drm2* and *cmt3* mutants show increased digestion with N but no effect on digestion with P. In (C), genomic DNA precut with the methylation-insensitive enzyme BstNI (B) was further digested with Psp1406I (P) indicative for CpG methylation, SacII (S) indicative for CpG/CpNpG methylation, and NheI (N) indicative for CpNpG/CpHpH methylation. B digestion ("−" lanes) yields one band (marked by an arrow), which is completely digested by P, S, and N in the unsilenced state (first panel). In presence of the silencer (second panel), cleavage of P and S is completely blocked due to acquired methylation, while N cleavage is partially inhibited. The *drm1 drm2* double mutant and *drm1 drm2 cmt3* triple mutant show increased digestion with N.

(D) RT-PCR expression analysis of the *NOSpro:NTPII* locus compared to the *ACTIN* locus in different mutant backgrounds. To control for DNA contamination, the reactions were performed either in the presence (RT+) or absence (RT−) of reverse transcriptase. The amounts of cDNA added to each reaction were adjusted so that an equal amount of *ACTIN* was amplified in each sample, except that the amount of cDNA added to the KK positive control reaction was reduced in order to maintain samples in a linear range of amplification. The *drm1 drm2* double mutant is included as a negative control because it also contains NPTII genes associated with the T-DNAs present in the *DRM1* and *DRM2* genes. However, PCR primers were designed to specifically detect the *NOSpro:NPTII* transgene.

ferases [17]. Previous work showed that a double mutant of *drm1* and *drm2* showed a lack of de novo DNA methylation normally associated with transgene silencing of the *FWA* and *SUPERMAN* genes [18]. It was also observed that *drm1 drm2* double-mutant plants show major losses of asymmetric methylation and more subtle and locus-specific effects on CpNpG methylation at endogenous *Arabidopsis* loci [12]. In this study, we have studied the roles of the *DRM* and *CMT3* genes in both the maintenance and initiation of RdDM in the H/K system.

DRM and *CMT3* Redundantly Control the Maintenance of RNA-Directed Non-CpG Methylation

To study the role of *DRM* and *CMT3* in the maintenance of RdDM in the H/K system, we crossed a *drm1 drm2 cmt3* triple-mutant plant to a line homozygous for both the H and K transgenes (HHKK). F1 plants were allowed to self pollinate, and F2 progeny plants were screened using PCR-based molecular markers to identify lines with the following genotypes: HHKK (with no methyltransferase mutations), HHKK *drm1 drm2*, HHKK *cmt3*, and HHKK *drm1 drm2 cmt3*. These F2 plants were allowed to self pollinate, and DNA was extracted from the F3 plants for methylation analysis. We used bisulfite genomic sequencing to measure the levels of DNA methylation at the *NOSpro:NTPII* target locus in each of these genotypes. Because the H and K transgenes were together before crossing in the methyltransferase mutations, this experiment measures the effect of the methyltransferase mutations on the maintenance of preexisting RdDM.

We found that CpG methylation of *NOSpro:NTPII* was

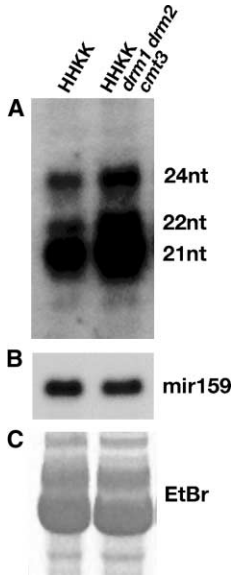

Figure 2. Northern Blot of Small RNAs Hybridized with a Sense NOSpro RNA Probe

(A) Approximately 21, 22, and 24 nucleotide (nt) siRNAs were detected in both HHKK and HHKK *drm1 drm2 cmt3* plants.

(B) Blot was stripped and reprobed with microRNA 159 as a loading control.

(C) The ethidium bromide (EtBr) staining pattern of the gel prior to blotting, to confirm that equal amounts of small RNAs were loaded in each lane.

not reduced in the *drm1 drm2*, *cmt3*, or *drm1 drm2 cmt3* triple-mutant plants (Figure 1A). These results are consistent with *MET1* functioning as the primary maintenance methyltransferase for CpG sites. At CpNpG sites, *drm1 drm2* double mutants and *cmt3* single mutants showed little reduction in DNA methylation. However, in the *drm1 drm2 cmt3* triple mutant, CpNpG methylation was lost. Thus, *DRM* and *CMT3* act redundantly to maintain RNA-directed CpNpG methylation. For cytosines in asymmetric sequence contexts, the *drm1 drm2* plants showed a major loss of methylation, while *cmt3* single-mutant plants did not show a reduction. However, the residual 3% of asymmetric methylation remaining in *drm1 drm2* double mutants was eliminated in the *drm1 drm2 cmt3* triple-mutant plants. Thus, *DRM* and *CMT3* also act redundantly to maintain RNA-directed asymmetric methylation.

Southern blot analysis using methylation-sensitive restriction enzymes was used to confirm the results obtained by bisulfite sequencing. We analyzed both the *NOSpro:NPTII* (Figure 1B) and the *NOSpro:NOS* (Figure 1C) genes present at the target K locus. We found that none of the *drm1 drm2*, *cmt3*, or *drm1 drm2 cmt3* mutant combinations had an effect on digestion with Psp1406I or SacII, which are sensitive to CpG methylation in their recognition sites (Figures 1B and 1C), confirming that *DRM* and *CMT3* do not affect CpG methylation. To assay non-CpG methylation we utilized NheI, which is blocked by methylation of the second cytosine in its recognition site GCTAG**C**. Because of the sequence context of the NheI site in the NOSpro sequence CA[GCTAGC]AA, this cytosine is in an asymmetric context on one strand and a CpNpG context on the other strand. Furthermore, NheI activity is only completely blocked by methylation of cytosines on both strands, meaning that loss of the asymmetric methylation on one strand or loss of the CpNpG methylation on the other strand can result in enzyme cleavage (Fermentas Life Sciences, http://www.fermentas.com/profiles/re/pnhei.htm). In plants containing the K target transgene but not the H silencing transgene, this site is cut completely by NheI, showing that this site is not methylated (Figures 1B and 1C). In plants containing both the H and K transgenes, NheI digestion is partially blocked, showing partial methylation of the site. The *cmt3* mutation showed only partially increased digestion of the NheI site at the *NOSpro:NPTII* gene and no detectable effect on NheI digestion at the *NOSpro:NOS* gene. However, both the *drm1 drm2* double mutant and the *drm1 drm2 cmt3* triple mutant showed complete digestion by NheI. These results are consistent with the bisulfite sequencing data, suggesting that the *DRM* genes are more critical than *CMT3* in maintaining RNA-directed non-CpG methylation.

The *drm* and *cmt3* Mutants Partially Release Preestablished RNA-Directed Transcriptional Gene Silencing

To test if *CMT3* plays a role in the maintenance of transcriptional gene silencing of the K target transgene, we crossed a *cmt3–7* homozygote to a plant homozygous for H and K. The F1 plant was allowed to self pollinate and 940 F2 seeds were tested for double antibiotic resistance on plates containing both hygromycin (20 mg/L) and kanamycin (40 mg/L). Hygromycin selects for the presence of the H transgene, and kanamycin resistance indicates activity of the *NOSpro:NTPII* gene. We obtained no plants showing double antibiotic resistance. We also tested 100 seeds of the HHKK *cmt3* line described above and again did not observe double antibiotic resistance. Thus, *cmt3* does not release transcriptional gene silencing of the *NOSpro:NTPII* gene to a level sufficient for kanamycin resistance. We were unable to perform a similar experiment with *drm1 drm2* double mutants, because the T-DNAs present in the *DRM1* and *DRM2* genes also confer kanamycin resistance. Therefore, we used reverse transcription polymerase chain reaction (RT-PCR) to measure the levels of *NPTII* mRNA produced in the HHKK, HHKK *drm1 drm2*, HHKK *cmt3*, and HHKK *drm1 drm2 cmt3* lines described above (Figure 1D). We found that the *drm1 drm2* double mutant and the *drm1 drm2 cmt3* triple mutant showed a partial reactivation of expression from the *NOSpro:NPTII* target gene. However, this level of expression was much lower than in target K plants that did not contain the H silencing transgene (KK lane in Figure 1D). The *cmt3* single mutant also showed some reactivation of *NOSpro:NPTII* but at an even lower level than in the *drm1 drm2* double mutant. These data suggest that *drm*, and to a lesser extent *cmt3*, can weakly reactivate NOS promoter activity. We also tested each of these genotypes for the activity of the *NOSpro:NOS* gene, by measuring levels of nopaline in tissue extracts [19]. We did not detect a significant level of nopaline in the HHKK *drm1 drm2*, HHKK *cmt3*, or HHKK *drm1 drm2 cmt3* lines, though nopaline was

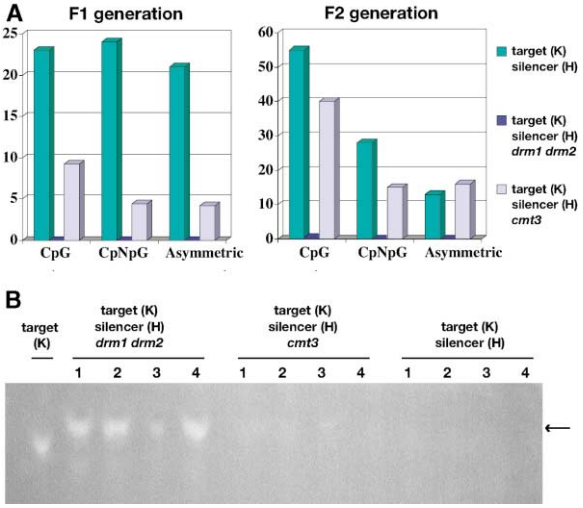

Figure 3. Effect of the *drm* and *cmt3* Mutations on Initiation of RdDM

(A) Methylation profiles of different mutants at the *NOSpro:NPTII* target locus, in either the F1 or F2 generations after crossing together plants homozygous for either the H or K transgenes. Histograms represent the percent methylation found in different sequence contexts as determined by bisulfite sequencing (details in Table S1).

(B) Results of nopaline assays on leaves of individual plants of the listed genotypes, which are described in the text. The position of nopaline is marked with an arrow. 1, 2, 3, and 4 represent four individual plants of the listed genotype.

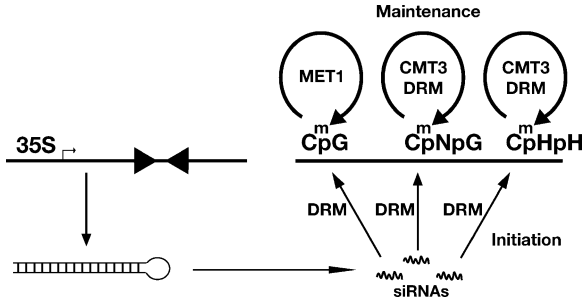

Figure 4. Proposed Functions of the DRM, CMT3, and MET1 Methyltransferases in the Initiation and Maintenance of RdDM

Double-stranded RNA and siRNAs produced from a hairpin transgene cause RdDM of unlinked DNA sequences. The *DRM* methyltransferase genes are required for the establishment of RNA-directed cytosine DNA methylation in all sequence contexts. Maintenance of RdDM is accomplished by MET1, which maintains CpG methylation, and CMT3 and DRM, which redundantly maintain CpNpG and asymmetric methylation (here designated CpHpH).

detectable in the KK positive control lines (data not shown). The nopaline detection assay employed here is likely not as sensitive as RT-PCR, such that weak reactivation of NOS promoter activity may not produce detectable levels of nopaline. In summary, the *drm* and *cmt3* mutants cause only a very weak reactivation of NOS promoter activity, presumably because the remaining CpG methylation in these mutants can largely maintain gene silencing.

DRM and *CMT3* Act Downstream of siRNA Accumulation

To study the relationship between siRNAs and the function of DNA methyltransferase genes, we measured the steady-state levels of siRNAs in HHKK or HHKK *drm1 drm2 cmt3* plants. We could readily detect NOSpro siRNAs in both genotypes (Figure 2). As shown previously [6, 20], three size classes of siRNAs were observed: an abundant species of 21 nucleotides in length, and two less abundant species of 22 and 24 nucleotides. It has been suggested that 21 and 22 nucleotide siRNAs are involved in posttranscriptional gene silencing, while the longer 24–26 nucleotide siRNAs are involved in the targeting of chromatin modifications, since only the longer species are associated with silenced endogenous retrotransposable elements, and the loss of these siRNAs is correlated with the loss of DNA and histone methylation [21, 22]. Interestingly, NOSpro siRNAs of all size classes were increased in abundance in the *drm1 drm2 cmt3* triple-mutant background (Figure 2). This result is similar to that of *hda6/rts1* mutants, which were isolated as suppressors of transcriptional silencing in the H/K system and which also increase siRNA abundance [20]. Thus, mutations affecting transcriptional gene silencing can cause feedback upregulation of siRNA accumulation. We also conclude that neither non-CpG methylation nor the DRM1, DRM2, or CMT3 proteins are required for continued accumulation of siRNAs generated in the H/K system.

DRM Is Required for the Initial Establishment of RdDM at CpG, CpNpG, and Asymmetric Sites

We tested whether *drm1 drm2* or *cmt3* mutants would block the initiation of DNA methylation of the target K transgene that normally occurs when the H and K transgenes are first brought together in a cross. It was previously shown that RdDM can occur within one generation of exposure of the target K transgene to the silencer H transgene [6]. Thus, we constructed lines homozygous for either *drm1 drm2* or *cmt3* in the H or K backgrounds. We then crossed an HH *drm1 drm2* plant with a KK *drm1 drm2* plant, an HH *cmt3* plant with a KK *cmt3* plant, and as a control we crossed an HH plant with a KK plant. In the F1 generation of these crosses, we examined the methylation level of the *NOSpro:NPTII* target gene by bisulfite genomic sequencing. Four individual plants of each genotype were analyzed in order to reduce sampling error, and the data were pooled from these samples. Since the target NOSpro has no methylation before exposure to the silencer H transgene, all of the methylation observed in the F1 generation will represent de novo RdDM. In the H/−K/− double hemizygous control plants, the NOSpro region became methylated in both symmetrical (CpG and CpNpG) and asymmetrical sites (Figure 3A). In the *cmt3* H/−K/− plants, methylation was present in all sequence contexts but was significantly lower than in the H/−K/− control plants. However, in the *drm1 drm2* H/−K/− F1 plants, no methylation was observed in any sequence context. This suggests that the *DRM* genes are required for the establishment of RdDM in the H/K system. We also analyzed the F2 progeny resulting from self pollina-

tion of the F1 plants, by using molecular markers to identify plants containing both the H and K transgenes. Four plants of each genotype were subjected to bisulfite sequencing, and pooled results are presented in Figure 3A. In the F2 H/K control plants, we observed higher levels of CpG methylation than in F1 plants, showing that full establishment of CpG methylation is progressive. In the *cmt3* homozygous plants, DNA methylation levels were similar to but slightly lower than the control. This suggests that while full levels of RdDM are delayed in the *cmt3* mutant, *CMT3* is not strictly required for establishment of RdDM in the H/K system. However, in the *drm1 drm2* homozygous plants, we did not detect methylation in any sequence context, again showing that DRM activity is required for the initiation of RdDM. Our findings with *CMT3* are in contrast to data in the *PAI* gene-silencing system where *cmt3* blocked the establishment of methylation of the *PAI2* gene caused by the inverted repeat *PAI1/PAI4* locus [23]. A possible explanation is that the higher levels of double-stranded RNA or siRNAs produced in the H/K system, relative to the *PAI* system [24], may overcome the requirement for *CMT3*.

To test whether the failure to establish DNA methylation in *drm1 drm2* plants was associated with a failure to establish transcriptional gene silencing, we performed nopaline assays in tissue extracts of each of the 16 F2 plants described above (Figure 3B). We found that the four H/K control plants and the four H/K *cmt3* plants did not produce a detectable level of nopaline, showing that the *NOSpro:NOS* gene was silent. However, in all four of the H/K *drm1 drm2* plants, we detected nopaline levels that were similar to the positive control plants that contained the K transgene but not the H transgene (Figure 3B). Therefore, the *DRM* genes are required for the establishment of RNA-directed transcriptional gene silencing at the *NOSpro:NOS* gene.

Role of Methyltransferase Genes in the Establishment and Maintenance of RdDM

Figure 4 summarizes the results of this study, showing the different methyltransferase requirements for the establishment and maintenance of RdDM in the H/K system. At CpG sites, the *DRM* genes are required for the establishment of methylation but not for the maintenance of preexisting methylation. Maintenance of CpG methylation can occur in the absence of triggering RNA signals and is dependent on the activity of *MET1* [6]. We cannot yet rule out that some CpG de novo methylation is catalyzed by MET1, but this would require cooperation with DRM activities. For CpNpG and asymmetric methylation, *DRM* genes are required for both the establishment and maintenance of methylation. *DRM* genes act redundantly with *CMT3* in their maintenance capacity, since CpNpG and asymmetric methylation are only totally lost when both types of genes are mutated. Previous work has shown that removing the source of double-stranded NOSpro RNA, either by segregating away the H transgene or by deleting the 35S promoter driving the hairpin RNA by cre-mediated recombination, results in a loss of non-CpG methylation in the H/K system [6]. Thus the "maintenance" phase described here for non-CpG methylation likely consists of persistent de novo activity of DRM and CMT3 in response to either double-stranded RNAs or siRNAs. This maintenance phase, however, can be clearly distinguished from the initiation phase, since *DRM* genes alone are strictly required for the latter.

The *DRM* and *CMT3* genes are required for non-CpG methylation at all loci that have been tested thus far, including endogenous genes, such as *SUPERMAN*, *FWA*, and *MEDEA* [12]; endogenous transposon sequences, such as *AtSN1*, *AtMu1*, and *Ta3* [12, 22]; and at the NOSpro sequences described here. In the H/K RdDM system there is a clear source of double-stranded RNA and siRNAs, which are required for the non-CpG methylation. In addition, there are indications that RNAs play a role in non-CpG methylation at endogenous loci as well. For instance, the *AtSN1* retrotransposable elements are associated with long (25 nucleotide) siRNAs, and the loss of these siRNAs correlates with a reduction of non-CpG *AtSN1* methylation [21, 22]. Furthermore, full levels of non-CpG methylation at *SUPERMAN*, *MEDEA*, *AtSN1*, and *AtMu1* depend on the activity of ARGONAUTE4, a type of protein normally associated with RNA interference and microRNA pathways [22]. Finally, in the *PAI* gene silencing system, non-CpG methylation of the *PAI2* locus depends on transcription of the inverted repeat containing PAI1–4 locus [24]. Thus, a likely possibility is that a large fraction non-CpG methylation is in some manner RNA directed.

Supplemental Material

Supplemental material, including an experimental procedures section and a table describing the detailed bisulfite genomic sequencing results, can be found at http://www.current-biology.com/cgi/content/full/13/24/2212/DC1.

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